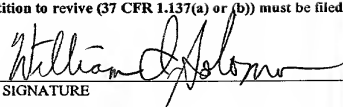


FORM PTO-1390) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV. 9-2001)		ATTORNEY'S DOCKET NUMBER <b>506.41066X00 Filed 1/7/02</b>
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) * CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>10/019957</b>
INTERNATIONAL APPLICATION NO. PCT/JP00/04535	INTERNATIONAL FILING DATE July 7, 2000	PRIORITY DATE CLAIMED July 7, 1999
TITLE OF INVENTION <b>PROCESS FOR PRODUCING HYDROXYLATED FATTY ACIDS AND <math>\delta</math>-LACTONES</b>		
APPLICANT(S) FOR DO/EO/US <b>SAITOH, CHIAKI; MASUDA, YUKIKO; YASHIRO, ATSUSHI; ISHIGURO, HIROKI</b>		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p>a. <input type="checkbox"/> is transmitted hereto (required only if not communicated by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office(RO/US)</p> <p>6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>a. <input checked="" type="checkbox"/> is attached hereto.</p> <p>b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p>a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p><b>Items 11 to 20 below concern document(s) or information included:</b></p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</p> <p>14. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input checked="" type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information: <b>PCT REQUEST FORM; INT'L. PUB. NO. WO01/04339-COVER SHEET; INT'L. SEARCH RPT.; CREDIT CARD PAYMENT FORM; Int'l. Prelim. Exam. Rpt.</b></p>		

U.S. APPLICATION NO. (If known, see 37 CFR 1.45) <b>10/019957</b>		INTERNATIONAL APPLICATION NO. PCT/JP0004535		ATTORNEY'S DOCKET NUMBER <b>506.41066X00</b>	
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<p>21. The following fees are submitted:</p> <p><b>BASIC NATIONAL FEE (37 CFR 1.492(a) (1) - (5)):</b></p> <p><input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....\$1040.00</p> <p><input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$890.00</p> <p><input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$740.00</p> <p><input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$710.00</p> <p><input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00</p> <p style="text-align: center;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></p> <p>Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(e)). <input type="checkbox"/> 20 <input type="checkbox"/> 30</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 15%;">CLAIMS</th> <th style="width: 15%;">NUMBER FILED</th> <th style="width: 15%;">NUMBER EXTRA</th> <th style="width: 15%;">RATE</th> <th style="width: 15%;">\$</th> <th style="width: 15%;"></th> </tr> <tr> <td>Total Claims</td> <td>81 - 20 =</td> <td>61</td> <td>x \$18.00</td> <td>\$1098.00</td> <td></td> </tr> <tr> <td>Independent Claims</td> <td>4 - 3 =</td> <td>1</td> <td>x \$84.00</td> <td>\$84.00</td> <td></td> </tr> <tr> <td colspan="4">MULTIPLE DEPENDENT CLAIMS(S) (if applicable)</td> <td>+ \$280.00</td> <td>\$</td> </tr> <tr> <td colspan="4"><b>TOTAL OF ABOVE CALCULATIONS =</b></td> <td><b>\$2072.00</b></td> <td></td> </tr> <tr> <td colspan="4"><input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.</td> <td>\$</td> <td></td> </tr> <tr> <td colspan="4"><b>SUBTOTAL =</b></td> <td><b>\$2072.00</b></td> <td></td> </tr> <tr> <td colspan="4">Processing fee of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30</td> <td>\$</td> <td></td> </tr> <tr> <td colspan="4"><b>TOTAL NATIONAL FEE =</b></td> <td><b>\$2072.00</b></td> <td></td> </tr> <tr> <td colspan="4">Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property</td> <td>\$40.00</td> <td></td> </tr> <tr> <td colspan="4"><b>TOTAL FEES ENCLOSED =</b></td> <td><b>\$2112.00</b></td> <td></td> </tr> <tr> <td colspan="4"></td> <td style="text-align: right;">Amount to be refunded:</td> <td>\$</td> </tr> <tr> <td colspan="4"></td> <td style="text-align: right;">charged:</td> <td>\$</td> </tr> </table> <p>a. <input type="checkbox"/> A check in the amount of \$_____ to cover the fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. <u>01-2135</u> in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposition Account No. <u>01-2135</u>. A duplicate copy of this sheet is enclosed.</p> <p>d. <input checked="" type="checkbox"/> Fees are to be charged to a credit card. <b>WARNING:</b> Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.</p> <p><b>NOTE:</b> Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</p> <p>SEND ALL CORRESPONDENCE TO:</p> <p>Antonelli, Terry, Stout &amp; Kraus, LLP 1300 North Seventeenth Street Suite 1800 Arlington, VA 22209 USA</p> <div style="text-align: right; margin-top: 20px;">       SIGNATURE      William I. Solomon      NAME      28,565      REGISTRATION NO.   </div>	CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$		Total Claims	81 - 20 =	61	x \$18.00	\$1098.00		Independent Claims	4 - 3 =	1	x \$84.00	\$84.00		MULTIPLE DEPENDENT CLAIMS(S) (if applicable)				+ \$280.00	\$	<b>TOTAL OF ABOVE CALCULATIONS =</b>				<b>\$2072.00</b>		<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$		<b>SUBTOTAL =</b>				<b>\$2072.00</b>		Processing fee of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30				\$		<b>TOTAL NATIONAL FEE =</b>				<b>\$2072.00</b>		Fee for recording the enclosed assignment (37 CFR 1.21(h)). 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506.41066X00

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant(s): Chiaki SAITOH ET AL.

Serial No.: New Application

Filed: Herewith

For: PROCESS FOR PRODUCING HYDROXYLATED FATTY  
ACIDS AND  $\delta$ -LACTONES

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

January 7, 2002

SIR:

Please amend the above-identified application, prior to calculating the  
filing fee, as follows:

**IN THE CLAIMS:**

Claims 3-10, 15-27 and 31-44 have been rewritten as indicated below:

3. (Amended) The process according to claim 1, wherein the first  
microorganism has the activity to introduce hydroxy into the 13-position and  
hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic  
acid to make the 12-position a single bond.

4. (Amended) The process according to claim 1, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

5. (Amended) The process according to claim 1, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

6. (Amended) The process according to claim 1, wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

7. (Amended) The process according to claim 1, wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IFO3778 or Bifidobacterium bifidum JCM7002.

8. (Amended) The process according to claim 1, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is 13-hydroxy-9-octadecenoic acid.

9. (Amended) The process according to claim 1, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linoleic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is 13-hydroxy-9,15-octadecadienoic acid.

10. (Amended) The process according to claim 1, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\gamma$ -linoleic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is 13-hydroxy-6,9-octadecadienoic acid.

15. (Amended) The process according to claim 12, wherein the double bond at the [n-6]-position is the cis-form.

16. (Amended) The process according to claim 12, wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

17. (Amended) The process according to claim 12, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

18. (Amended) The process according to claim 12, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

19. (Amended) The process according to claim 12, wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

20. (Amended) The process according to claim 12, wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IF03778 or Bifidobacterium bifidum JCM7002.

21. (Amended) The process according to claim 12, wherein the second microorganism is a yeast.

22. (Amended) The process according to claim 12, wherein the second microorganism belongs to the genus Kluyveromyces, Zygosaccharomyces, Pichia or Saccharomyces.

23. (Amended) The process according to claim 12, wherein the second microorganism is Kluyveromyces marxianus, Kluyveromyces thermotolerans, Kluyveromyces wickerhamii, Zygosaccharomyces rouxii, Zygosaccharomyces bailii, Zygosaccharomyces cidri, Pichia jadinii or Saccharomyces cerevisiae.

24. (Amended) The process according to claim 12, wherein the second microorganism is Kluyveromyces marxianus IFO1090, Kluyveromyces thermotolerans ATCC24177, Kluyveromyces wickerhamii ATCC24178, Zygosaccharomyces rouxii NFR2007, Zygosaccharomyces bailii ATCC8766, Zygosaccharomyces cidri ATCC46819, Pichia jadinii IFO0987 or Saccharomyces cerevisiae Kyokai No. 701.

25. (Amended) The process according to claim 12, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the  $\delta$ -lactone is  $\delta$ -decalactone.

26. (Amended) The process according to claim 12, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more)

wherein at least the [n-6]-position is a double bond is  $\alpha$ -linoleic acid and the  $\delta$ -lactone is jasmine lactone.

27. (Amended) The process according to claim 12, wherein the composition is a natural oil or fat or a hydrolyzate thereof.

31. (Amended) The process according to claim 28, wherein the double bond at the [n-6]-position is the cis-form.

32. (Amended) The process according to claim 28, wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

33. (Amended) The process according to claim 28, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

34. (Amended) The process according to claim 28, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.



35. (Amended) The process according to claim 28, wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

36. (Amended) The process according to claim 28, wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IF03778 or Bifidobacterium bifidum JCM7002.

37. (Amended) The process according to claim 28, wherein the second microorganism is a yeast.

38. (Amended) The process according to claim 28, wherein the second microorganism belongs to the genus Kluyveromyces, Zygosaccharomyces, Pichia or Saccharomyces.

39. (Amended) The process according to claim 28, wherein the second microorganism is Kluyveromyces marxianus, Kluyveromyces thermotolerans, Kluyveromyces wickerhamii, Zygosaccharomyces rouxii, Zygosaccharomyces bailii, Zygosaccharomyces cidri, Pichia jadinii or Saccharomyces cerevisiae.

40. (Amended) The process according to claim 28, wherein the second microorganism is Kluyveromyces marxianus IFO1090, Kluyveromyces thermotolerans ATCC24177, Kluyveromyces wickerhamii ATCC24178, Zygosaccharomyces rouxii NFR2007, Zygosaccharomyces bailii ATCC8766, Zygosaccharomyces cidri ATCC46819, Pichia jadinii IFO0987or Saccharomyces cerevisiae Kyokai No. 701.

41. (Amended) The process according to claim 28, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the  $\delta$ -lactone is  $\delta$ -decalactone.

42. (Amended) The process according to claim 28, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linoleic acid and the  $\delta$ -lactone is jasmine lactone.

43. (Amended) The process according to claim 28, wherein the composition is a food.

44. (Amended) The process for producing a food containing a  $\delta$ -lactone, which comprises adding to a food the  $\delta$ -lactone produced by the process according to claim 12.

Please add the following new claims:

45. The process for producing a food containing a  $\delta$ -lactone, which comprises adding to a food the composition containing the  $\delta$ -lactone produced by the process according to claim 28.

46. The process according to claim 2, wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

47. The process according to claim 46, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

48. The process according to claim 46, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

49. The process according to claim 46, wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

50. The process according to claim 46, wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IFO3778 or Bifidobacterium bifidum JCM7002.

51. The process according to claim 50, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is 13-hydroxy-9-octadecenoic acid.

52. The process according to claim 50, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linoleic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is 13-hydroxy-9,15-octadecadienoic acid.

53. The process according to claim 50, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\gamma$ -linoleic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is 13-hydroxy-6,9-octadecadienoic acid.

54. The process according to claim 14, wherein the double bond at the [n-6]-position is the cis-form.

55. The process according to claim 54, wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

56. The process according to claim 54, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

57. The process according to claim 54, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

58. The process according to claim 54, wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

59. The process according to claim 54, wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IF03778 or Bifidobacterium bifidum JCM7002.

60. The process according to claim 59, wherein the second microorganism is a yeast.

61. The process according to claim 59, wherein the second microorganism belongs to the genus Kluyveromyces, Zygosaccharomyces, Pichia or Saccharomyces.

62. The process according to claim 59, wherein the second microorganism is Kluyveromyces marxianus, Kluyveromyces thermotolerans, Kluyveromyces wickerhamii, Zygosaccharomyces rouxii, Zygosaccharomyces bailii, Zygosaccharomyces cidri, Pichia jadinii or Saccharomyces cerevisiae.

63. The process according to claim 59, wherein the second microorganism is Kluyveromyces marxianus IFO1090, Kluyveromyces thermotolerans ATCC24177, Kluyveromyces wickerhamii ATCC24178, Zygosaccharomyces rouxii NFR2007, Zygosaccharomyces bailii ATCC8766, Zygosaccharomyces cidri ATCC46819, Pichia jadinii IFO0987or Saccharomyces cerevisiae Kyokai No. 701.

64. The process according to claim 63, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the  $\delta$ -lactone is  $\delta$ -decalactone.

65. The process according to claim 63, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linoleic acid and the  $\delta$ -lactone is jasmine lactone.

66. The process according to claim 65, wherein the composition is a natural oil or fat or a hydrolyzate thereof.

67. The process according to claim 30, wherein the double bond at the [n-6]-position is the cis-form.

68. The process according to claim 67, wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

69. The process according to claim 67, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

70. The process according to claim 67, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

71. The process according to claim 67, wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

72. The process according to claim 67, wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IF03778 or Bifidobacterium bifidum JCM7002.



73. The process according to claim 72, wherein the second microorganism is a yeast.

74. The process according to claim 72, wherein the second microorganism belongs to the genus Kluyveromyces, Zygosaccharomyces, Pichia or Saccharomyces.

75. The process according to claim 72, wherein the second microorganism is Kluyveromyces marxianus, Kluyveromyces thermotolerans, Kluyveromyces wickerhamii, Zygosaccharomyces rouxii, Zygosaccharomyces bailii, Zygosaccharomyces cidri, Pichia jadinii or Saccharomyces cerevisiae.

76. The process according to claim 72, wherein the second microorganism is Kluyveromyces marxianus IFO1090, Kluyveromyces thermotolerans ATCC24177, Kluyveromyces wickerhamii ATCC24178, Zygosaccharomyces rouxii NFR2007, Zygosaccharomyces bailii ATCC8766, Zygosaccharomyces cidri ATCC46819, Pichia jadinii IFO0987or Saccharomyces cerevisiae Kyokai No. 701.

77. The process according to claim 76, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the  $\delta$ -lactone is  $\delta$ -decalactone.

78. The process according to claim 76, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linoleic acid and the  $\delta$ -lactone is jasmine lactone.

79. The process according to claim 78, wherein the composition is a food.

80. The process for producing a food containing a  $\delta$ -lactone, which comprises adding to a food the  $\delta$ -lactone produced by the process according to claim 66.

81. The process for producing a food containing a  $\delta$ -lactone, which comprises adding to a food the composition containing the  $\delta$ -lactone produced by the process according to claim 78.--

## **REMARKS**

Applicants have amended the original claims, prior to examination of the above-identified application and prior to calculating the filing fee, in order to delete multiple dependency, including improper multiple dependency in various of the claims. Moreover, Applicants have amended claim 44 to delete the alternative embodiments (and alternative dependency) recited in the same claim.

In light of amendments to previously submitted claims, Applicants are adding new claims 45-81 to the application. Claim 45 corresponds to claim 44, but includes therein use of the composition recited in the alternative embodiment set forth in original claim 44. Claims 46-81 have been added in light of the deletion of multiple dependency.

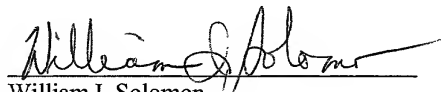
Entry of the preliminary amendments, and, subsequent thereto, calculation of the filing fee and then examination of the above-identified application, are respectfully requested.

Attached hereto is a marked-up version of the changes made to the claims by the current Preliminary Amendment. The changes are shown on the attached pages, the first page of which is captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE**".

To the extent necessary, applicants petition for an extension of time under 37 CFR §1.136. Please charge any shortage in the fees due in connection with the filing of this paper, including extension of time fees, to Deposit Account No. 01-2135 (506.41066X00) and please credit any excess fees to such deposit account.

Respectfully submitted,

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ATTACHMENT A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

Claims 3-10, 15-27 and 31-44 have been amended as indicated below:

3. (Amended) The process according to claim 1 [or 2], wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

4. (Amended) The process according to [any of claims 1 to 3] claim 1, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

5. (Amended) The process according to [any of claims 1 to 3] claim 1, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

6. (Amended) The process according to [any of claims 1 to 3] claim 1, wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

7. (Amended) The process according to [any of claims 1 to 3] claim 1, wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IFO3778 or Bifidobacterium bifidum JCM7002.

8. (Amended) The process according to [any of claims 1 to 7] claim 1, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is 13-hydroxy-9-octadecenoic acid.

9. (Amended) The process according to [any of claims 1 to 7] claim 1, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linoleic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is 13-hydroxy-9,15-octadecadienoic acid.

10. (Amended) The process according to [any of claims 1 to 7] claim 1, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\gamma$ -linoleic

acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is 13-hydroxy-6,9-octadecadienoic acid.

15. (Amended) The process according to [any of claims 12 to 14] claim 12, wherein the double bond at the [n-6]-position is the cis-form.

16. (Amended) The process according to [any of claims 12 to 15] claim 12, wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

17. (Amended) The process according to [any of claims 12 to 15] claim 12, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

18. (Amended) The process according to [any of claims 12 to 15] claim 12, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

19. (Amended) The process according to [any of claims 12 to 15] claim 12, wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

20. (Amended) The process according to [any of claims 12 to 15] claim 12, wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IF03778 or Bifidobacterium bifidum JCM7002.

21. (Amended) The process according to [any of claims 12 to 20] claim 12, wherein the second microorganism is a yeast.

22. (Amended) The process according to [any of claims 12 to 20] claim 12, wherein the second microorganism belongs to the genus Kluyveromyces, Zygosaccharomyces, Pichia or Saccharomyces.

23. (Amended) The process according to [any of claims 12 to 20] claim 12, wherein the second microorganism is Kluyveromyces marxianus, Kluyveromyces thermotolerans, Kluyveromyces wickerhamii, Zygosaccharomyces rouxii, Zygosaccharomyces bailii, Zygosaccharomyces cidri, Pichia jadinii or Saccharomyces cerevisiae.



24. (Amended) The process according to [any of claims 12 to 20] claim 12, wherein the second microorganism is Kluyveromyces marxianus IFO1090, Kluyveromyces thermotolerans ATCC24177, Kluyveromyces wickerhamii ATCC24178, Zygosaccharomyces rouxii NFR2007, Zygosaccharomyces bailii ATCC8766, Zygosaccharomyces cidri ATCC46819, Pichia jadinii IFO0987 or Saccharomyces cerevisiae Kyokai No. 701.

25. (Amended) The process according to [any of claims 12 to 24] claim 12, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the  $\delta$ -lactone is  $\delta$ -decalactone.

26. (Amended) The process according to [any of claims 12 to 24] claim 12, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linoleic acid and the  $\delta$ -lactone is jasmine lactone.

27. (Amended) The process according to [any of claims 12 to 26] claim 12, wherein the composition is a natural oil or fat or a hydrolyzate thereof.

31. (Amended) The process according to [any of claims 28 to 30] claim 28, wherein the double bond at the [n-6]-position is the cis-form.

32. (Amended) The process according to [any of claims 28 to 31] claim 28, wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

33. (Amended) The process according to [any of claims 28 to 31] claim 28, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

34. (Amended) The process according to [any of claims 28 to 31] claim 28, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

35. (Amended) The process according to [any of claims 28 to 31] claim 28, wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

36. (Amended) The process according to [any of claims 28 to 31] claim 28, wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IF03778 or Bifidobacterium bifidum JCM7002.

37. (Amended) The process according to [any of claims 28 to 36] claim 28, wherein the second microorganism is a yeast.

38. (Amended) The process according to [any of claims 28 to 36] claim 28, wherein the second microorganism belongs to the genus Kluyveromyces, Zygosaccharomyces, Pichia or Saccharomyces.

39. (Amended) The process according to [any of claims 28 to 36] claim 28, wherein the second microorganism is Kluyveromyces marxianus, Kluyveromyces thermotolerans, Kluyveromyces wickerhamii, Zygosaccharomyces rouxii, Zygosaccharomyces bailii, Zygosaccharomyces cidri, Pichia jadinii or Saccharomyces cerevisiae.

40. (Amended) The process according to [any of claims 28 to 36] claim 28, wherein the second microorganism is Kluyveromyces marxianus IFO1090, Kluyveromyces thermotolerans ATCC24177, Kluyveromyces wickerhamii

ATCC24178, Zygosaccharomyces rouxii NFR2007, Zygosaccharomyces bailii ATCC8766, Zygosaccharomyces cidri ATCC46819, Pichia jadinii IFO0987 or Saccharomyces cerevisiae Kyokai No. 701.

41. (Amended) The process according to [any of claims 28 to 40] claim 28, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the  $\delta$ -lactone is  $\delta$ -decalactone.

42. (Amended) The process according to [any of claims 28 to 40] claim 28, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linoleic acid and the  $\delta$ -lactone is jasmine lactone.

43. (Amended) The process according to [any of claims 28 to 42] claim 28, wherein the composition is a food.

44. (Amended) The process for producing a food containing a  $\delta$ -lactone, which comprises adding to a food the  $\delta$ -lactone produced by the process

Year	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100
1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	

## SPECIFICATION

PROCESS FOR PRODUCING HYDROXYLATED FATTY ACIDS AND  $\delta$ -LACTONESTechnical Field

- 5           The present invention relates to a process for producing [n-5]-hydroxy fatty acids (n is an even number of 10 or more) wherein the [n-6]-position is a single bond from straight-chain fatty acids having n carbon atoms wherein at least the [n-6]-position is a double bond. The present invention also  
10 relates to a process for producing  $\delta$ -lactones from the fatty acids and compositions containing  $\delta$ -lactones.

Background Art

- 15           Lactones are important compounds which are widely used as food additives because of their property of imparting preferable flavors such as a fruit flavor and a milk flavor. However, as lactones are contained in natural materials only at low concentrations, chemically synthesized products are generally used.
- 20           As for the process for producing lactones using microorganisms, it is known that 10-hydroxyoctadecanoic acid, a precursor of  $\gamma$ -dodecalactone, is formed from oleic acid by the activity of microorganisms such as lactic acid bacteria and bifidobacteria [Gastroenterology, 62, 430 (1972)],  
25 bacteria belonging to the genus Corynebacterium [Agricultural and Biological Chemistry, 45, 2025 (1981)] and bacteria belonging to the genus Pseudomonas [Archives of Biochemistry and Biophysics, 99, 249 (1962)]. It is also known that hydroxylated fatty acids such as 10-hydroxyoctadecanoic acid  
30 and ricinoleic acid in castor oil can be converted into  $\gamma$ -lactones by the activity of yeasts (Japanese Published Unexamined Patent Application Nos. 66991/85 and 100508/85).
- Sporobolomyces odor, a yeast which produces lactones, is known to produce  $\delta$ -decalactone from linoleic acid. From  
35 the fact that  $\delta$ -decalactone is produced from 13-hydroxy-9Z,11E-octadecadienoic acid (coriolic acid), it is

presumed that the yeast produces coriolic acid as an intermediate [ACS SYMPOSIUM SERIES, Flavor Precursors, 490, 46 (1992)].

A method is known for converting coriolic acid as a precursor obtained by reduction of hydroperoxide obtained by subjecting linoleic acid to photooxidation or treatment with soybean lipoxygenase into  $\delta$ -decalactone by the activity of bacteria belonging to the genus Cladosporium or yeasts (Japanese Published Unexamined Patent Application No. 187387/91). Also known is a method for converting coriolic acid contained in seed oil of Coriaria nepalensis and 11-hydroxypalmitic acid extracted from the roots of Mexican jalap as precursors into  $\delta$ -decalactone by the activity of bacteria belonging to the genus Cladosporium [Journal of Organic Chemistry, 54, 4979 (1989)] or yeasts [Journal of Organic Chemistry, 57, 1954 (1992)] and Japanese Published Unexamined Patent Application No. 219886/91].

However, there has not been reported a process for producing  $\delta$ -decalactone and jasmine lactone respectively from 13-hydroxy-9-octadecenoic acid and 13-hydroxy-9,15-octadecadienoic acid formed respectively from linoleic acid and  $\alpha$ -linolenic acid by the activity of microorganisms.

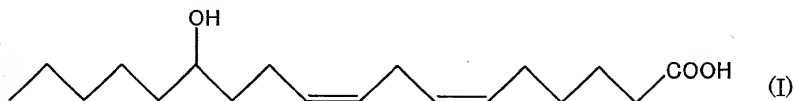
#### Disclosure of the Invention

An object of the present invention is to provide a process for producing [n-5]-hydroxy fatty acids (n is an even number of 10 or more) wherein the [n-6]-position is a single bond from straight-chain fatty acids having n carbon atoms wherein at least the [n-6]-position is a double bond using a microorganism. Another object of the present invention is to provide a process for producing  $\delta$ -lactones from the fatty acids using a microorganism and to provide compositions containing  $\delta$ -lactones.

The present invention relates to a process for producing an [n-5]-hydroxy fatty acid (n is an even number of 10 or more) wherein the [n-6]-position is a single bond, which comprises

causing cells or a culture of a microorganism having the activity to introduce hydroxy into the [n-5]-position and hydrogen into the [n-6]-position of a straight-chain fatty acid having n carbon atoms wherein at least the [n-6]-position is a double bond to make the [n-6]-position a single bond (hereinafter referred to as the first microorganism) or a treated matter thereof to act on a straight-chain fatty acid having n carbon atoms wherein at least the [n-6]-position is a double bond or a composition containing the fatty acid to form the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond, and recovering the formed [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond.

The present invention also relates to 13-hydroxy-6,9-octadecadienoic acid represented by the following formula (I):



The present invention also relates to a process for producing a  $\delta$ -lactone, which comprises causing cells or a culture of the first microorganism or a treated matter thereof to act on a straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond or a composition containing the fatty acid to form an [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond, then causing cells or a culture of a microorganism having the activity to  $\beta$ -oxidize an [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond (hereinafter referred to as the second microorganism) or a treated matter thereof to act on the formed [n-5]-hydroxy fatty acid, and recovering the formed  $\delta$ -lactone.

The present invention also relates to a process for producing a composition containing a  $\delta$ -lactone, which comprises causing cells or a culture of the first microorganism or a treated



matter thereof to act on a composition containing a straight-chain fatty acid having  $n$  carbon atoms ( $n$  is an even number of 10 or more) wherein at least the  $[n-6]$ -position is a double bond to form an  $[n-5]$ -hydroxy fatty acid wherein the

5  $[n-6]$ -position is a single bond in the composition, and then causing cells or a culture of the second microorganism or a treated matter thereof to act on the formed  $[n-5]$ -hydroxy fatty acid.

The present invention further relates to a process for

10 producing a food containing a  $\delta$ -lactone, which comprises adding the  $\delta$ -lactones or the composition containing the  $\delta$ -lactones produced by the above process to a food.

In the present invention, the expression "the  $[n-m]$ -position is a double bond" means that the  $[n-m]$ -position

15 and the  $[n-(m-1)]$ -position are bonded by a double bond.

The straight-chain fatty acids having  $n$  carbon atoms ( $n$  is an even number of 10 or more) wherein at least the  $[n-6]$ -position is a double bond of the present invention include monoenoic fatty acids, dienoic fatty acids and trienoic fatty

20 acids wherein the  $[n-6]$ -position is a double bond, preferably a *cis*-form double bond.

Preferred polyenoic fatty acids (e.g., dienoic fatty acids and trienoic fatty acids) are nonconjugated polyenoic fatty acids wherein the  $[n-9]$ -position is a double bond,

25 specifically a *cis*-form double bond. When  $n$  is 12 or more, fatty acids wherein the  $[n-10]$ - and lower positions are single bonds are desirable for the formation of  $\delta$ -lactones.

There is no upper limit to  $n$  insofar as  $n$  is an even number of 10 or more, but  $n$  is preferably 10 to 32, more preferably

30 12 to 26, and most preferably 16 to 22.

Examples of the monoenoic fatty acids include decenoic acid, dodecenoic acid, tetradecenoic acid, hexadecenoic acid, octadecenoic acid, eicosenoic acid, docosenoic acid, tetracosenoic acid, hexacosenoic acid, octacosenoic acid,

35 triacontenoic acid, dotriacontenoic acid and tetratriacontenoic acid.

Examples of the dienoic fatty acids include decadienoic acid, dodecadienoic acid, tetradecadienoic acid, hexadecadienoic acid, octadecadienoic acid, eicosadienoic acid, docosadienoic acid, tetracosadienoic acid, hexacosadienoic acid, octacosadienoic acid, triacontadienoic acid, dotriacontadienoic acid and tetratriacontadienoic acid.

Examples of the trienoic fatty acids include decatrienoic acid, dodecatrienoic acid, tetradecatrienoic acid, hexadecatrienoic acid, octadecatrienoic acid, eicosatrienoic acid, docosatrienoic acid, tetracosatrienoic acid, hexacosatrienoic acid, octacosatrienoic acid, triacontatrienoic acid, dotriacontatrienoic acid and tetratriacontatrienoic acid.

Specific examples of these fatty acids include 4-decenoic acid, 7,10-hexadecadienoic acid, 6,10-hexadecadienoic acid, 12-octadecenoic acid, linoleic acid,  $\alpha$ -linolenic acid,  $\gamma$ -linolenic acid, 11,14-eicosadienoic acid, 5,11,14-eicosatrienoic acid, 8,11,14-eicosatrienoic acid, bishomo- $\gamma$ -linolenic acid, 11,14,17-eicosatrienoic acid, 6,9,12,15-octadecatetraenoic acid, 13,16-docosadienoic acid, 7,10,13,16-docosatetraenoic acid and arachidonic acid. Preferred are linoleic acid,  $\alpha$ -linolenic acid and  $\gamma$ -linolenic acid, and more preferred are linoleic acid and  $\alpha$ -linolenic acid.

In the present invention, a composition containing the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond can also be used. Such compositions include natural oils and fats, foods and hydrolyzates thereof.

Examples of the natural oils and fats include obtusiloba oil, evening primrose seed oil, soybean oil, corn oil, safflower oil, wheat germ oil, rice oil, sesame oil, rapeseed oil, olive oil, linseed oil, milk fat, suet, lard, egg yolk oil, fish oil, seaweed, algae, filamentous fungi, ferns and protozoa.

Examples of the foods include foods, such as soybean milk, prepared by adding the straight-chain fatty acids having n carbon

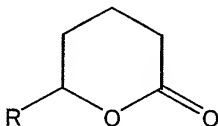
atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond to foods which do not contain the fatty acids, besides foods containing the straight-chain fatty acids having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond.

Hydrolyzates of natural oils and fats or foods can be obtained by treating natural oils and fats or foods with hydrolase, etc.

An example of the hydrolase is lipase.

There is no specific limit as to the amount of the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond contained in the composition, but it is preferably 0.01 to 99 wt%, more preferably 0.1 to 90 wt%.

The  $\delta$ -lactones of the present invention include  $\delta$ -lactones represented by the following formula (II):



(II)

(wherein R represents n-pentyl or n-pentenyl), for example,  $\delta$ -decalactone and jasmine lactone.

As the first microorganism of the present invention, any microorganism can be used that has the activity to introduce hydroxy into the [n-5]-position and hydrogen into the [n-6]-position of a straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond, preferably a cis-form double bond, to make the [n-6]-position a single bond. Preferred is a microorganism which has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

Examples of the first microorganism include lactic acid

bacteria and bifidobacteria. Lactic acid bacteria include those belonging to the genus Pediococcus, e.g. Pediococcus pentosaceus. Bifidobacteria include those belonging to the genus Bifidobacterium, e.g. Bifidobacterium bifidum.

- 5 Specifically, Pediococcus pentosaceus IFO3891, Pediococcus sp. IFO3778, Bifidobacterium bifidum JCM7002, etc. are preferably used.

As the second microorganism of the present invention, any microorganism can be used that has the activity to  $\beta$ -oxidize an [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond. For example, yeasts can be used.

Examples of suitable yeasts include microorganisms belonging to the genera Kluyveromyces, Zygosaccharomyces, Pichia and Saccharomyces.

- 15 Examples of the microorganisms belonging to the genus Kluyveromyces are Kluyveromyces marxianus, Kluyveromyces thermotolerans and Kluyveromyces wickerhamii. Examples of the microorganisms belonging to the genus Zygosaccharomyces are Zygosaccharomyces rouxii, Zygosaccharomyces bailii and Zygosaccharomyces cidri. An example of the microorganism belonging to the genus Pichia is Pichia jadinii. An example of the microorganism belonging to the genus Saccharomyces is Saccharomyces cerevisiae. As second microorganism, for example, Kluyveromyces marxianus IFO1090, Kluyveromyces
- 20 thermotolerans ATCC24177, Kluyveromyces wickerhamii ATCC24178, Zygosaccharomyces rouxii NFR2007, Zygosaccharomyces bailii ATCC8766, Zygosaccharomyces cidri ATCC46819, Pichia jadinii IFO0987, Saccharomyces cerevisiae Kyokai No. 701 (sake yeast), etc. are preferably used.

- 30 These microorganisms may be used alone or in combination.

In the present invention, any mutants of the above microorganisms obtained by artificial mutation methods such as ultraviolet irradiation, X-ray irradiation, treatment with mutagens and gene manipulation, or by spontaneous mutation may also be used as long as they are microorganisms having the above-described activities.

35

As the medium for the culturing of these microorganisms, any synthetic or natural medium containing carbon sources, nitrogen sources, inorganic substances and trace components that can be used for general culturing of lactic acid bacteria, bifidobacteria or yeast can be used.

Examples of the carbon sources include starch, dextrin, sucrose, glucose, mannose, fructose, raffinose, rhamnose, inositol, lactose, xylose, arabinose, mannitol, molasses and pyruvic acid. They may be used alone or in combination, preferably in an amount of 1 to 20 g/l.

Examples of the nitrogen sources include ammonium salts such as ammonium chloride, ammonium sulfate, ammonium phosphate, ammonium carbonate and ammonium acetate, nitrates such as sodium nitrate and potassium nitrate, and nitrogen-containing organic substances such as peptone, yeast extract, meat extract, malt extract, corn steep liquor, casein hydrolyzate, soybean powder, vegetable juice, casamino acid and urea. They may be used alone or in combination, preferably in an amount of 1 to 20 g/l.

Examples of the inorganic substances include sodium chloride, potassium chloride, calcium chloride, magnesium sulfate, calcium carbonate, dipotassium hydrogenphosphate, potassium dihydrogenphosphate, ferrous sulfate, calcium chloride, manganese sulfate, zinc sulfate and copper sulfate. They may be used alone or in combination, preferably in an amount of 0.1 to 2 g/l.

Examples of the trace components include vitamins such as biotin, thiamine and nicotinic acid, and amino acids such as  $\beta$ -alanine and glutamic acid. They may be used alone or in combination, preferably in an amount of 0.0001 to 2 g/l.

As the method of culturing, liquid culture, especially submerged spinner culture is preferred. The medium is adjusted to pH 2 to 11, preferably pH 3 to 10, more preferably pH 4 to 8. Culturing is carried out at 10 to 80°C, preferably 10 to 60°C, most preferably 20 to 40°C generally for 6 hours to 7 days. The pH of the medium is adjusted with aqueous ammonia, an ammonium carbonate solution, etc.

The treated matters of the culture used in the present invention include cells of a microorganism having the activity to introduce hydroxy into the [n-5]-position and hydrogen into the [n-6]-position of a straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond to make the [n-6]-position a single bond, cultures containing the microorganism and treated matters thereof.

The treated matters of the cells of the microorganism include dried cells, frozen cells, refrigerated cells, freeze-dried cells, heated cells, compressed cells, ultrasonically disrupted cells, products obtained by treating the cells with a surfactant, an organic solvent or a lytic enzyme, immobilized cells, and enzymes obtained from the cells by means of extraction or purification.

An enzyme can be extracted and purified from the cells according to general methods for extraction and purification of a protein. For example, an enzyme can be extracted from the cells using a homogenizer or glass beads, or by ammonia dissolution, the enzyme method, etc., and then purified by means of filtration, centrifugation, salting-out, precipitation with an organic solvent, immune precipitation, etc. as well as dialysis, ultrafiltration, gel filtration, electrophoresis, chromatography using an adsorbent, an affinity adsorbent or molecular sieves, liquid-phase partition, ion exchange, batch method and crystallization, alone or in combination.

As the culture containing the microorganism, a culture obtained after the completion of the culturing can be used as such. Also useful are treated matters obtained by treating the culture by means of concentration, drying, freezing, refrigeration, freeze-drying, heating, pressing, ultrasonic disruption, treatment with a surfactant, an organic solvent or a lytic enzyme, etc. alone or in combination.

The process for producing an [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond and the process for producing a  $\delta$ -lactone are described below.

The [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond can be produced by causing cells or a culture of the first microorganism or a treated matter thereof to act on a straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond or a composition containing the fatty acid, and recovering the formed [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond. Details of the process are as follows.

The cells or culture of the first microorganism or a treated matter thereof is subjected to reaction with the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond or a composition containing the fatty acid at 10 to 80°C, preferably 20 to 40°C, at pH 2 to 11, preferably pH 3 to 10, more preferably at pH 5 to 8, for 6 hours to 7 days, preferably 1 to 4 days. If necessary, an aqueous medium such as water may be added prior to the reaction.

A buffer, a surfactant, an organic solvent, an antioxidant, etc. may be added to the reaction mixture as may be required.

Examples of the buffer include phosphate buffer and citrate buffer. The concentration of the buffer is preferably 0.01 to 1 mol/l.

Examples of the surfactant include sucrose fatty acid ester, sorbitan fatty acid ester and glycerin fatty acid ester. The concentration of the surfactant is preferably 0.1 to 5%.

An example of the organic solvent is ethanol. The concentration of the organic solvent is preferably 1 to 50 g/l.

Examples of the antioxidant include those applicable to foods, such as  $\alpha$ -tocopherol, vitamin E, butylhydroxyanisole (BHA), dibutylhydroxytoluene (BHT) and skim milk powder. The concentration of the antioxidant is preferably 0.01 to 50 g/l.

When the cells of the microorganism are used, reaction is carried out in the following manner. Into 5 to 50 ml of a medium containing carbon sources, nitrogen sources, etc. are inoculated 1 to 3 loopfuls of the cells of the first microorganism,

followed by stationary culture for 1 to 5 days. The obtained seed culture is inoculated in an amount of 0.1 to 5% into a straight-chain fatty acid having  $n$  carbon atoms ( $n$  is an even number of 10 or more) wherein at least the  $[n-6]$ -position is a double bond or a composition containing the fatty acid, followed by stationary culture or culturing with stirring at a low speed. Culturing may be carried out at any temperature that allows conversion of the straight-chain fatty acid into an  $[n-5]$ -hydroxy fatty acid wherein the  $[n-6]$ -position is a single bond, preferably 5 to 40°C. It does not matter whether the first microorganism grows or not by the culturing. The culturing time varies depending upon the conditions, but it is usually 1 to 4 days.

Extraction and detection of the  $[n-5]$ -hydroxy fatty acid ( $n$  is an even number of 10 or more) wherein the  $[n-6]$ -position is a single bond converted from the straight-chain fatty acid having  $n$  carbon atoms wherein at least the  $[n-6]$ -position is a double bond and contained in the reaction mixture or culture can be carried out according to a conventional method of lipid extraction and a method of lipid detection by thin layer chromatography (TLC). That is, to about 0.2 to 10 ml of the reaction mixture is added about 30 to 80 wt% solvent such as chloroform/methanol (2:1, v/v). After 10 minutes of shaking, the resulting mixture is centrifuged to obtain a solvent layer as a lipid extract. The obtained lipid extract (1 to 20  $\mu$ l) is spotted on a silica gel-precoated TLC plate and developed using an appropriate solvent system, followed by coloration using an appropriate color developer. The  $[n-5]$ -hydroxy fatty acid wherein the  $[n-6]$ -position is a single bond can be detected by the coloration on the plate.

As the TLC plate, TLC glass plate 60 (No. 5721, Merck & Co., Inc.), or the like may be used.

The  $[n-5]$ -hydroxy fatty acid wherein the  $[n-6]$ -position is a single bond can be isolated and purified from the reaction mixture or culture by conventional methods for isolation and purification of lipid. That is, the  $[n-5]$ -hydroxy fatty acid



wherein the [n-6]-position is a single bond can be isolated and purified by carrying out steps such as removal of cells by filtration, centrifugation, etc., extraction of lipid by the use of a solvent such as diethyl ether/toluene (15:85 to 5 60:40, v/v), adsorption and desorption of lipid by column chromatography or thin layer chromatography using adsorbent resin, silica gel, reversed-phase silica gel, aluminum oxide, cellulose, diatomaceous earth, magnesium silicate, gel filtering agent, ion exchange resin, etc., and partition using 10 an appropriate solvent system. The purity of the obtained fatty acid is about 90 to 100%.

The [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond can be detected by thin layer chromatography according to the above-described method.

15 The [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond can be determined, for example, by high performance liquid chromatography under the following conditions.

20 Apparatus: SPD-10A (Shimadzu Corporation)

Column: TSK-gel ODS-80Ts (Tosoh Corporation)

Mobile phase: solution A: acetonitrile/water/acetic acid  
(28:72:0.02, v/v/v)

solution B: acetonitrile/water/acetic acid  
25 (52:48:0.02, v/v/v)

solution A (10 minutes), solution A → solution B (60 minutes,  
linear concentration gradient), solution B (30 minutes)

Flow rate: 2 ml/minute

Temperature: 40°C

30 Detection: UV-200 nm

The [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond can be used for production of a  $\delta$ -lactone which is useful as food additives, etc.

35 The process for producing a  $\delta$ -lactone from the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single

bond is described below.

The cells or culture of the second microorganism or a treated matter thereof is subjected to reaction with a reaction mixture containing the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond produced by the above-described process, a treated matter of the reaction mixture or the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond isolated from the reaction mixture at 10 to 80°C, preferably 20 to 50°C, at pH 2 to 9, preferably pH 3 to 8, more preferably pH 4 to 7, for 12 hours to 7 days, preferably 1 to 4 days, most preferably 2 to 3 days. If necessary, an aqueous medium such as water may be added prior to the reaction.

The "treated matter of the reaction mixture containing the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond" refers to a treated matter containing the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond obtained in the process of isolation and purification of the reaction mixture containing the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond.

The above-described buffer, surfactant, organic solvent, antioxidant, etc. may be added to the reaction mixture as may be required.

When the cells of the microorganism are used, reaction is carried out in the following manner. A seed culture obtained by inoculating 1 to 3 loopfuls of the cells of the second microorganism into 5 to 50 ml of a medium containing carbon sources, nitrogen sources, etc., followed by stationary culture for 1-5 days, or another seed culture obtained by inoculating the above seed culture in an amount of 1 to 5% into 100 ml to 1 l of a medium, followed by stationary culture for 1 to 5 days, is inoculated in an amount of 0.1 to 50% into the reaction mixture containing the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond, followed by culturing under aerobic conditions, e.g., with aeration and stirring. There is no specific restriction as to the conditions of aeration and stirring, but it is preferred to carry out aeration at 0.01

to 3 vvm and stirring at 200 to 1200 rpm. Culturing may be carried out at any temperature that allows conversion of the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond into a  $\delta$ -lactone, preferably 5 to 35°C. It does not matter whether the microorganism grows or not by the culturing. The culturing time varies depending upon the conditions, but it is usually 12 hours to 7 days.

After the completion of the reaction with the second microorganism or the culturing of the second microorganism, the reaction mixture or culture may be adjusted to pH 1 to 6, preferably pH 3 to 5, and further subjected to reaction at 5 to 80°C, preferably 20 to 35°C for 30 minutes or more to increase the yield of  $\delta$ -lactone. This method is preferably employed when Saccharomyces cerevisiae, Kluyveromyces marxianus, etc. are used as the second microorganism.

The  $\delta$ -lactone can be isolated and purified from the reaction mixture or culture by conventional methods such as extraction with a solvent. That is, the  $\delta$ -lactone can be isolated and purified by adding about 20 to 60 wt% pentane/ether mixture (5:95 to 80:20, v/v) and 20 to 60 wt% saturated aqueous solution of sodium chloride to about 0.2 to 10 ml of the reaction mixture or culture, followed by shaking for 10 minutes, and then centrifuging the resulting mixture to obtain the supernatant.

The  $\delta$ -lactones can be determined, for example, by gas chromatography under the following conditions.

Apparatus: Gas chromatograph mass spectrometer GCMS-QP5000  
(Shimadzu Corporation)

Column: TC-WAX 60 m 0.25 mm x 0.25  $\mu$ m

Helium flow rate: 0.5 ml/minute

Column temperature: 40°C (0.5 minute)-5°C/minute-240°C  
(69.5 minutes)

Pressure: 50 Kpa (0 minute)-5Kpa/minute-300 Kpa (60 minutes)

Standard  $\delta$ -lactone preparation:  $\delta$ -decalactone (Aldrich),  
jasmine lactone (Nippon Zeon)

The reaction mixture or culture containing the  $\delta$ -lactone obtained by the above-described process can be added to foods, etc., as such, or if necessary, after being sterilized or after  
 5 removing solids therefrom by filtration. The purified  $\delta$ -lactone can also be added to foods.

The  $\delta$ -lactones may be added to any foods, preferably, milk beverages, processed milk products, processed animal food products, confectionery (e.g., cakes, ice cream and snacks)  
 10 and seasonings (e.g., white sauces, cheese sauces and dressings).

The  $\delta$ -lactones are usually added to foods at a concentration of ca. 0.1 to 100 ppm, preferably ca. 0.25 to 20 ppm.

15 Examples, Comparative Examples and Test Examples of the present invention are described below.

#### Best Modes for Carrying Out the Invention

In the following Examples, the measurement of FAB mass spectrum and high resolution FAB mass spectrum was carried out  
 20 using JMS-HX/HX110A (JEOL Ltd.), and the NMR measurement was carried out using JNM-A400 (JEOL Ltd.) according to a conventional method.

#### 25 Example 1 Formation of hydroxy fatty acids

Linoleic acid,  $\gamma$ -linolenic acid,  $\alpha$ -linolenic acid, cis-11,cis-14-eicosadienoic acid,  
 cis-8,cis-11,cis-14-eicosatrienoic acid,  
 cis-11,cis-14,cis-17-eicosatrienoic acid,  
 30 cis-13,cis-16-docosadienoic acid and cis-12-octadecenoic acid (0.5 g each, all produced by Sigma Chemical Co.) were respectively added to 100 ml of a nutrient medium comprising 0.18 g of yeast extract, 0.42 g of polypeptone and 0.62 ml of 60% liquid glucose (pH 6.5). To each of the resulting mixtures were added 0.02  
 35 ml of E-mix 80 (Eisai Co., Ltd.) as an antioxidant and 2 g of skim milk powder as a dispersing agent, and 3 ml of a seed culture

of Pediococcus pentosaceus IF03891 was inoculated therein, followed by culturing with stirring at 80 rpm at 25°C for 2 days.

To 0.5 ml of each of the resulting cultures was added an equal amount of a chloroform/methanol mixture (2:1, v/v) as a solvent to extract lipid. The obtained lipid extract was spotted on TLC glass plate silica gel 60 (No. 5721, Merck & Co., Inc.) in 5  $\mu$ l portions. The first development was carried out using toluene/diethyl ether/ethanol/acetic acid (50:40:2:0.2, v/v/v/v) for 20 minutes, followed by drying of the plate, and the second development was carried out using hexane/diethyl ether (94:6, v/v) for 35 minutes, followed by drying of the plate. As a color developer, an appropriate amount of 8% (w/w) solution of phosphoric acid containing 6 g/100 ml copper acetate was sprayed on the development surface, followed by heating at 140°C for 25 minutes.

The results are shown in Table 1.

Table 1

Fatty acid	Rf value
Linoleic acid	0.19
$\gamma$ -Linolenic acid	0.17
$\alpha$ -Linolenic acid	0.16
cis-11,cis-14-Eicosadienoic acid	0.18
cis-8,cis-11,cis-14-Eicosatrienoic acid	0.18
cis-11,cis-14,cis-17-Eicosatrienoic acid	0.18
cis-13,cis-16-Docosadienoic acid	0.20
cis-12-Octadecenoic acid	0.19

As shown in Table 1, with every one of the fatty acids used, a hydroxy fatty acid was detected as a brown spot at an Rf value in the range of 0.13 to 0.22, which is the Rf value range expected for hydroxy fatty acids.

#### Example 2 Production of a hydroxide of linoleic acid

Linoleic acid (5 g) was added to 1000 ml of a nutrient

medium comprising 1.8 g of yeast extract, 4.2 g of polypeptone and 6.2 ml of 60% liquid glucose (pH 6.5), and 0.2 ml of E-mix 80 as an antioxidant and 20 g of skim milk powder as a dispersing agent were added thereto. Into the resulting mixture was  
 5 inoculated 30 ml of a seed culture of Pediococcus pentosaceus IFO3891, followed by culturing with stirring at 80 rpm at 25°C for 2 days.

To ca. 1000 ml of the obtained culture was added ca. 120 wt% diethyl ether/toluene (4:5, v/v), followed by shaking for  
 10 about 20 minutes, and the resulting mixture was centrifuged to separate the supernatant as a lipid extract. After the lipid extract was concentrated to ca. 50 ml with a rotary evaporator, column chromatography was carried out using a glass column having an inside diameter of 3.14 cm x 50 cm packed with ca. 80 g of  
 15 silica gel (Wako gel C-2000) in the following manner.

After the column was washed with 300 ml of the above solvent mixture, the concentrated lipid extract was added thereto. Then, 500 ml of the above solvent mixture was passed through the column at a flow rate of 3 ml/minute and the eluate was taken in 5  
 20 ml fractions. The lipid in each fraction was developed on a silica gel-precoated TLC plate, whereby 13-hydroxy-9-octadecenoic acid was detected and obtained.

[Physicochemical properties of 13-hydroxy-9-octadecenoic  
 25 acid]

- (1) Molecular formula:  $C_{18}H_{34}O_3$
- (2) FAB mass spectrum:  $m/z$  299 (M+H)<sup>+</sup>
- (3) High resolution FAB mass spectrum:  $m/z$  299.2592 (M+H)<sup>+</sup>,  
 Calculated for  $C_{18}H_{35}O_3$ : 299.2586
- 30 (4) <sup>13</sup>C-NMR spectrum (100 MHz, CDCl<sub>3</sub>):  $\delta$  ppm (multiplicity),  
 179.2(s), 130.5(d), 129.3(d), 71.9(d), 37.4(t), 37.3(t),  
 34.0(t), 31.9(t), 29.7(t), 28.9(t), 28.9(t), 28.9(t),  
 27.1(t), 25.3(t), 24.7(t), 23.6(t), 22.6(t), 14.0(q)
- (5) <sup>1</sup>H-NMR spectrum (400 MHz, CDCl<sub>3</sub>):  $\delta$  ppm [integration,  
 35 multiplicity, coupling constant J (Hz)], 5.38(1H, m),  
 5.38(1H, m), 3.63(1H, m), 2.33(2H, t, 7.4), 2.12(2H, m),

2.04(2H, q, 6.5), 1.63(2H, m), 1.52(2H, m), 1.52(2H, m),  
1.44(2H, m), 1.31(2H, m), 1.31(2H, m), 1.31(2H, m), 1.31(2H,  
m), 1.31(2H, m), 1.31(2H, m), 0.89(3H, t, 6.8)

5 Example 3 Production of a hydroxide of  $\alpha$ -linolenic acid (13-hydroxy-9,15-octadecadienoic acid)

The same procedure as in Example 2 was repeated, except that linoleic acid was replaced by  $\alpha$ -linolenic acid, whereby 13-hydroxy-9,15-octadecadienoic acid was obtained.

10

[Physicochemical properties of  
13-hydroxy-9,15-octadecadienoic acid]

(1) Molecular formula:  $C_{18}H_{32}O_3$

(2) FAB mass spectrum:  $m/z$  297 (M+H)<sup>+</sup>

15 (3) High resolution FAB mass spectrum:  $m/z$  297.2421 (M+H)<sup>+</sup>,  
Calculated for  $C_{18}H_{33}O_3$ : 297.2430

(4)  $^{13}C$ -NMR spectrum (100 MHz,  $CDCl_3$ ):  $\delta$  ppm (multiplicity),  
178.3(s), 135.2(d), 130.6(d), 129.2(d), 124.4(d), 71.3(d),  
36.7(t), 35.3(t), 33.9(t), 29.5(t), 28.9(t), 28.9(t),  
20 28.9(t), 27.1(t), 24.7(t), 23.7(t), 20.7(t), 14.3(q)

(5)  $^1H$ -NMR spectrum (400 MHz,  $CDCl_3$ ):  $\delta$  ppm [integration,  
multiplicity, coupling constant J (Hz)], 5.56(1H, m),  
5.38(1H, m), 5.37(1H, m), 5.37(1H, m), 3.65(1H, m), 2.34(2H,  
t, 7.4), 2.23(2H, t, 7.1), 2.15(2H, m), 2.07(2H, m), 2.03(2H,  
25 m), 1.64(2H, m, 7.1), 1.54(2H, m), 1.32(2H, m), 1.32(2H,  
m), 1.32(2H, m), 1.32(2H, m), 0.97(3H, t, 7.4)

Example 4 Production of a hydroxide of  $\gamma$ -linolenic acid  
(13-hydroxy-6,9-octadecadienoic acid)

30 The same procedure as in Example 2 was repeated, except  
that linoleic acid was replaced by  $\gamma$ -linolenic acid, whereby  
13-hydroxy-6,9-octadecadienoic acid was obtained.

[Physicochemical properties of 13-hydroxy-6,9-  
35 octadecadienoic acid]

(1) Molecular formula:  $C_{18}H_{32}O_3$

- (2) FAB mass spectrum:  $m/z$  297 ( $M+H$ )<sup>+</sup>  
 (3) High resolution FAB mass spectrum:  $m/z$  297.2426 ( $M+H$ )<sup>+</sup>,  
 Calculated for  $C_{18}H_{33}O_3$ : 297.2430  
 (4)  $^{13}C$ -NMR spectrum (100 MHz,  $CDCl_3$ ):  $\delta$ ppm (multiplicity),  
 178.6(s), 129.6(d), 129.4(d), 128.4(d), 128.4(d), 71.8(d),  
 37.4(t), 37.1(t), 33.9(t), 31.9(t), 28.9(t), 26.8(t),  
 25.6(t), 25.3(t), 24.4(t), 23.6(t), 22.6(t), 14.0(q)  
 (5)  $^1H$ -NMR spectrum (400 MHz,  $CDCl_3$ ):  $\delta$ ppm [integration,  
 multiplicity, coupling constant  $J$  (Hz)], 5.38(1H, m),  
 5.38(1H, m), 5.38(1H, m), 3.64(2H, m), 2.80(2H,  
 t, 5.6), 2.35(2H, t, 7.3), 2.18(2H, m), 2.09(2H, q, 7.5),  
 1.67(2H, m), 1.55(2H, m), 1.45(2H, m), 1.45(2H, m), 1.42(2H,  
 m), 1.31(2H, m), 1.31(2H, m), 0.89(3H, t, 6.8)

#### 15 Example 5

Linoleic acid (0.5 g) was added to 100 ml of a nutrient medium comprising 0.18 g of yeast extract, 0.42 g of polypeptone and 0.62 ml of 60% liquid glucose (pH 6.5), and 0.02 ml of E-mix 80 as an antioxidant and 2 g of skim milk powder as a dispersing agent were added thereto. Into the resulting mixture was inoculated 3 ml of a seed culture of Pediococcus pentosaceus IFO3891 as the first microorganism, followed by culturing with stirring at 80 rpm at 25°C for 2 days.

To 0.5 ml of the resulting culture was added an equal amount of a chloroform/methanol mixture (2:1, v/v) as a solvent to extract lipid. The obtained lipid extract was spotted on TLC glass plate silica gel 60 in 5  $\mu$ l portions. The first development was carried out using toluene/diethyl ether/ethanol/acetic acid (50:40:2:0.2, v/v/v/v) for 20 minutes, followed by drying of the plate, and the second development was carried out using hexane/diethyl ether (94:6, v/v) for 35 minutes, followed by drying of the plate. As a color developer, an appropriate amount of 8% (w/w) solution of phosphoric acid containing 6 g/100 ml copper acetate was sprayed on the development surface, followed by heating at 140°C for 25 minutes.



As a result, a hydroxy fatty acid converted from linoleic acid was detected as a brown spot at the position of 0.19 of Rf value.

5 Example 6

$\alpha$ -Linolenic acid (0.5 g) was added to 100 ml of a nutrient medium comprising 0.18 g of yeast extract, 0.42 g of polypeptone and 0.62 ml of 60% liquid glucose (pH 6.5), and 0.02 ml of E-mix 80 as an antioxidant and 2 g of skim milk powder as a dispersing agent were added thereto. Into the resulting mixture was inoculated 3 ml of a seed culture of Pediococcus pentosaceus IFO3891 as the first microorganism, followed by culturing with stirring at 80 rpm at 25°C for 2 days.

15 After the completion of the culturing, extraction of lipid from the culture and thin layer chromatography were carried out in the same manner as in Example 1 to detect a hydroxy fatty acid converted from  $\alpha$ -linolenic acid.

As a result, the hydroxy fatty acid was detected as a brown spot at the position of 0.16 of Rf value.

20

Example 7

$\gamma$ -Linolenic acid (0.5 g) was added to 100 ml of a nutrient medium comprising 0.18 g of yeast extract, 0.42 g of polypeptone and 0.62 ml of 60% liquid glucose (pH 6.5), and 0.02 ml of E-mix 80 as an antioxidant and 2 g of skim milk powder as a dispersing agent were added thereto. Into the resulting mixture was inoculated 3 ml of a seed culture of Pediococcus pentosaceus IFO3891 as the first microorganism, followed by culturing with stirring at 80 rpm at 25°C for 2 days.

30 After the completion of the culturing, extraction of lipid from the culture and thin layer chromatography were carried out in the same manner as in Example 1 to detect a hydroxy fatty acid converted from  $\gamma$ -linolenic acid.

35 As a result, the hydroxy fatty acid was detected as a brown spot at the position of 0.17 of Rf value.

Example 8

Linoleic acid (5 g) was added to 1 l of a nutrient medium comprising 1.8 g of yeast extract, 4.2 g of polypeptone and 6.2 ml of 60% liquid glucose (pH 6.5), and 0.2 ml of E-mix 80 as an antioxidant and 20 g of skim milk powder as a dispersing agent were added thereto. Into the resulting mixture was inoculated 30 ml of a seed culture of Pediococcus pentosaceus IFO3891, followed by culturing with stirring at 400 rpm at 25°C for 2 days.

After the completion of the culturing, 30 ml of a seed culture of Kluyveromyces marxianus IFO1090 was inoculated into the obtained culture, followed by culturing with stirring at 900 rpm under aeration at 1 vvm at 25°C for 4 days.

As a result, 245 ppm of  $\delta$ -decalactone was obtained in the culture.

Example 9

$\alpha$ -Linolenic acid (5 g) was added to 1 l of a nutrient medium comprising 1.8 g of yeast extract, 4.2 g of polypeptone and 6.2 ml of 60% liquid glucose (pH 6.5), and 0.2 ml of E-mix 80 as an antioxidant and 20 g of skim milk powder as a dispersing agent were added thereto. Into the resulting mixture was inoculated 30 ml of a seed culture of Pediococcus pentosaceus IFO3891, followed by culturing with stirring at 400 rpm at 25°C for 2 days.

After the completion of the culturing, 30 ml of a seed culture of Kluyveromyces marxianus IFO1090 was inoculated into the obtained culture, followed by culturing with stirring at 900 rpm under aeration at 1 vvm at 25°C for 4 days.

As a result, 103 ppm of jasmine lactone was obtained in the culture.

Example 10

To 930 ml of water was added 40 ml of corn oil (Ajinomoto Co., Inc.), and 0.2 ml of E-mix 80 as an antioxidant and 0.4 g of Lipase MY (Meito Sangyo Co., Ltd.) as a hydrolase were

added thereto. The mixture was allowed to stand at 40°C for 24 hours to cause hydrolysis. To the obtained hydrolyzed corn oil were added 20 g of skim milk powder, 1.8 g of yeast extract, 4.2 g of polypeptone and 6.2 ml of 60% liquid glucose, and the resulting mixture was adjusted to pH 6.5. Into the resulting mixture was inoculated 30 ml of a seed culture of Pediococcus pentosaceus IFO3891, followed by culturing with stirring at 400 rpm at 25°C for 2 days.

After the completion of the culturing, 30 ml of a seed culture of Kluyveromyces marxianus IFO1090 was inoculated into the obtained culture, followed by culturing with stirring at 900 rpm under aeration at 1 vvm at 25°C for 4 days.

As a result, 392 ppm of  $\delta$ -decalactone was obtained in the culture.

#### Example 11

A seed culture of Pediococcus sp. IFO3778 (30 ml) was inoculated into a medium containing hydrolyzed corn oil prepared in the same manner as in Example 10, followed by culturing with stirring at 400 rpm at 25°C for 2 days.

After the completion of the culturing, 30 ml of a seed culture of Kluyveromyces marxianus IFO1090 was inoculated into the obtained culture, followed by culturing with stirring at 900 rpm under aeration at 1 vvm at 25°C for 4 days.

As a result, 173 ppm of  $\delta$ -decalactone was obtained in the culture.

#### Example 12

A seed culture of Bifidobacterium bifidum JCM7002 (30 ml) was inoculated into a medium containing hydrolyzed corn oil prepared in the same manner as in Example 10, followed by culturing with stirring at 400 rpm at 25°C for 2 days.

After the completion of the culturing, 30 ml of a seed culture of Kluyveromyces marxianus IFO1090 was inoculated into the obtained culture, followed by culturing with stirring at 900 rpm under aeration at 1 vvm at 25°C for 4 days.

As a result, 115 ppm of  $\delta$ -decalactone was obtained in the culture.

#### Example 13

5 A seed culture of Pediococcus pentosaceus IFO3891 (30 ml) was inoculated into a medium containing hydrolyzed corn oil prepared in the same manner as in Example 10, followed by culturing with stirring at 400 rpm at 25°C for 2 days.

10 After the completion of the culturing, 30 ml of a seed culture of Saccharomyces cerevisiae Kyokai No. 701 was inoculated into the obtained culture, followed by culturing with stirring at 900 rpm under aeration at 1 vvm at 25°C for 4 days.

15 As a result, 23 ppm of  $\delta$ -decalactone was obtained in the culture. To the culture was added 90% lactic acid (Musashino Kagaku Kenkyusho Co., Ltd.) to adjust the culture to pH 3, and the resulting mixture was allowed to stand at 25°C for 30 minutes for further reaction. As a result, 197 ppm of  $\delta$ -decalactone was obtained in the reaction mixture.

#### Example 14

20 A seed culture of Pediococcus pentosaceus IFO3891 (30 ml) was inoculated into a medium containing hydrolyzed corn oil prepared in the same manner as in Example 10, followed by culturing with stirring at 400 rpm at 25°C for 2 days.

After the completion of the culturing, 30 ml of a seed culture of Kluyveromyces marxianus IFO1090 was inoculated into the obtained culture, followed by culturing with stirring at 900 rpm under aeration at 1 vvm at 25°C for 4 days.

30 As a result, 378 ppm of  $\delta$ -decalactone was obtained in the culture. To the culture was added 90% lactic acid to adjust the culture to pH 3, and the resulting mixture was allowed to stand at 25°C for 30 minutes for further reaction.

35 As a result, 783 ppm of  $\delta$ -decalactone was obtained in the reaction mixture.

### Example 15

To 465 ml of water was added 20 ml of corn oil (Ajinomoto Co., Inc.), and 0.1 ml of E-mix 80 as an antioxidant and 0.2 g of Lipase MY (Meito Sangyo Co., Ltd.) as a hydrolase were added thereto. The mixture was allowed to stand at 40°C for 24 hours to cause hydrolysis. To the obtained hydrolyzed corn oil were added 10 g of skim milk powder, 0.9 g of yeast extract, 2.1 g of polypeptone and 3.1 ml of 60% liquid glucose, and the resulting mixture was adjusted to pH 6.5. Into the resulting mixture was inoculated 15 ml of a seed culture of Pediococcus pentosaceus IF03891, followed by culturing with stirring at 400 rpm at 25°C for 2 days.

After the completion of the culturing, the obtained culture was mixed with 500 ml of a seed culture of Kluyveromyces marxianus IF01090 obtained by culturing in a nutrient medium for 2 days, and the resulting mixture was cultured with stirring at 900 rpm under aeration at 1 vvm at 25°C for 2 days.

As a result, 502 ppm of  $\delta$ -decalactone was obtained in the culture.

### Example 16

The culture obtained in Example 10 was sterilized at 85°C for one minute, and 0.6 ml of the sterilized culture was added to 200 g of commercially available corn cream soup. The concentration of  $\delta$ -decalactone in the soup was 1.18 ppm. As a result of the addition of  $\delta$ -decalactone, corn cream soup having a mild milky taste was obtained.

### Example 17

The culture obtained in Example 10 was sterilized at 85°C for one minute, and 0.3 ml of the sterilized culture was added to 200 ml of commercially available low fat milk. The concentration of  $\delta$ -decalactone in the low fat milk was 0.59 ppm. As a result of the addition of  $\delta$ -decalactone, the smell of milk powder was masked and low fat milk having an improved milk flavor was obtained.

Example 18

Into 500 ml of soybean milk was inoculated 15 ml of Pediococcus pentosaceus IFO3891 as a lactic acid bacterium, followed by stationary culture at 25°C for one day.

After the completion of the culturing, 15 ml of Kluyveromyces marxianus IFO1090 was inoculated into the obtained culture, followed by culturing with stirring at 900 rpm under aeration at 1 vvm at 25°C for 2 days.

As a result, 2.7 ppm of  $\delta$ -decalactone was obtained in the culture. Additionally, a grassy smell of the culture was masked and fermented soybean milk having an improved flavor was obtained.

Industrial Applicability

The present invention provides a process for producing [n-5]-hydroxy fatty acids (n is an even number of 10 or more) wherein the [n-6]-position is a single bond from straight-chain fatty acids having n carbon atoms wherein at least the [n-6]-position is a double bond. The present invention also provides a process for producing  $\delta$ -lactones from straight-chain fatty acids having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond via [n-5]-hydroxy fatty acids wherein the [n-6]-position is a single bond. The present invention further provides a process for producing compositions containing  $\delta$ -lactones from compositions containing straight-chain fatty acids having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond.

In accordance with the present invention, industrially useful  $\delta$ -lactones can be readily produced in large amounts from fatty acids derived from inexpensive food materials such as linoleic acid,  $\alpha$ -linolenic acid and  $\gamma$ -linolenic acid using cells or a culture of a microorganism or a treated matter thereof.

## CLAIMS

1. A process for producing an [n-5]-hydroxy fatty acid (n is an even number of 10 or more) wherein the [n-6]-position is a single bond, which comprises causing cells or a culture of a microorganism having the activity to introduce hydroxy into the [n-5]-position and hydrogen into the [n-6]-position of a straight-chain fatty acid having n carbon atoms wherein at least the [n-6]-position is a double bond to make the [n-6]-position a single bond (hereinafter referred to as the first microorganism) or a treated matter thereof to act on a straight-chain fatty acid having n carbon atoms wherein at least the [n-6]-position is a double bond or a composition containing the fatty acid to form the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond, and recovering the formed [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond.

2. The process according to claim 1, wherein the double bond at the [n-6]-position is the cis-form.

3. The process according to claim 1 or 2, wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

4. The process according to any of claims 1 to 3, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

5. The process according to any of claims 1 to 3, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

6. The process according to any of claims 1 to 3, wherein

the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

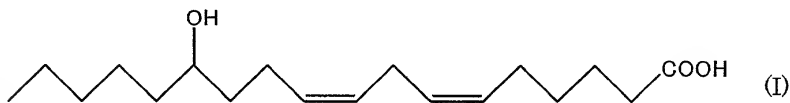
7. The process according to any of claims 1 to 3, wherein  
5 the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IFO3778 or Bifidobacterium bifidum JCM7002.

8. The process according to any of claims 1 to 7, wherein  
10 the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is 13-hydroxy-9-octadecenoic acid.

15 9. The process according to any of claims 1 to 7, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linolenic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is  
20 13-hydroxy-9,15-octadecadienoic acid.

10. The process according to any of claims 1 to 7, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\gamma$ -linolenic acid and the [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond is  
25 13-hydroxy-6,9-octadecadienoic acid.

11. 13-Hydroxy-6,9-octadecadienoic acid represented by  
30 the following formula (I):

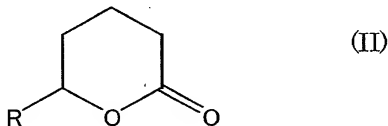


12. A process for producing a  $\delta$ -lactone, which comprises



causing cells or a culture of the first microorganism or a treated matter thereof to act on a straight-chain fatty acid having  $n$  carbon atoms ( $n$  is an even number of 10 or more) wherein at least the [n-6]-position is a double bond or a composition containing the fatty acid to form an [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond, causing cells or a culture of a microorganism having the activity to  $\beta$ -oxidize an [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond (hereinafter referred to as the second microorganism) or a treated matter thereof to act on the formed [n-5]-hydroxy fatty acid, and recovering the formed  $\delta$ -lactone.

13. The process according to claim 12, wherein the  $\delta$ -lactone is a  $\delta$ -lactone represented by the following formula (II):



(wherein R represents n-pentyl or n-pentenyl).

14. The process according to claim 12, wherein the  $\delta$ -lactone is  $\delta$ -decalactone or jasmine lactone.

15. The process according to any of claims 12 to 14, wherein the double bond at the [n-6]-position is the cis-form.

16. The process according to any of claims 12 to 15, wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

17. The process according to any of claims 12 to 15,

wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

18. The process according to any of claims 12 to 15,  
5 wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

19. The process according to any of claims 12 to 15,  
10 wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

20. The process according to any of claims 12 to 15,  
15 wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IFO3778 or Bifidobacterium bifidum JCM7002.

21. The process according to any of claims 12 to 20,  
wherein the second microorganism is a yeast.

22. The process according to any of claims 12 to 20,  
20 wherein the second microorganism belongs to the genus Kluyveromyces, Zygosaccharomyces, Pichia or Saccharomyces.

23. The process according to any of claims 12 to 20,  
25 wherein the second microorganism is Kluyveromyces marxianus, Kluyveromyces thermotolerans, Kluyveromyces wickerhamii, Zygosaccharomyces rouxii, Zygosaccharomyces bailii, Zygosaccharomyces cidri, Pichia jadinii or Saccharomyces cerevisiae.

24. The process according to any of claims 12 to 20,  
30 wherein the second microorganism is Kluyveromyces marxianus IFO1090, Kluyveromyces thermotolerans ATCC24177, Kluyveromyces wickerhamii ATCC24178, Zygosaccharomyces rouxii NFR2007, Zygosaccharomyces bailii ATCC8766, Zygosaccharomyces cidri ATCC46819, Pichia jadinii IFO0987 or Saccharomyces

cerevisiae Kyokai No. 701.

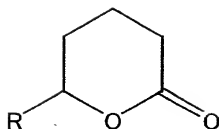
25. The process according to any of claims 12 to 24, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the  $\delta$ -lactone is  $\delta$ -decalactone.

26. The process according to any of claims 12 to 24, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linolenic acid and the  $\delta$ -lactone is jasmine lactone.

27. The process according to any of claims 12 to 26, wherein the composition is a natural oil or fat or a hydrolyzate thereof.

28. A process for producing a composition containing a  $\delta$ -lactone, which comprises causing cells or a culture of the first microorganism or a treated matter thereof to act on a composition containing a straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond to form an [n-5]-hydroxy fatty acid wherein the [n-6]-position is a single bond in the composition, and then causing cells or a culture of the second microorganism or a treated matter thereof to act on the formed [n-5]-hydroxy fatty acid.

29. The process according to claim 28, wherein the  $\delta$ -lactone is a  $\delta$ -lactone represented by the following formula (II):



(II)

(wherein R represents n-pentyl or n-pentenyl).

5           30. The process according to claim 29, wherein the  $\delta$ -lactone is  $\delta$ -decalactone or jasmine lactone.

10           31. The process according to any of claims 28 to 30, wherein the double bond at the [n-6]-position is the cis-form.

15           32. The process according to any of claims 28 to 31, wherein the first microorganism has the activity to introduce hydroxy into the 13-position and hydrogen into the 12-position of linoleic acid,  $\alpha$ -linolenic acid or  $\gamma$ -linolenic acid to make the 12-position a single bond.

20           33. The process according to any of claims 28 to 31, wherein the first microorganism is a lactic acid bacterium or bifidobacterium.

25           34. The process according to any of claims 28 to 31, wherein the first microorganism belongs to the genus Pediococcus or Bifidobacterium.

30           35. The process according to any of claims 28 to 31, wherein the first microorganism is Pediococcus pentosaceus or Bifidobacterium bifidum.

            36. The process according to any of claims 28 to 31, wherein the first microorganism is Pediococcus pentosaceus IFO3891, Pediococcus sp. IFO3778 or Bifidobacterium bifidum JCM7002.

37. The process according to any of claims 28 to 36, wherein the second microorganism is a yeast.

38. The process according to any of claims 28 to 36, wherein the second microorganism belongs to the genus Kluyveromyces, Zygosaccharomyces, Pichia or Saccharomyces.

39. The process according to any of claims 28 to 36, wherein the second microorganism is Kluyveromyces marxianus, Kluyveromyces thermotolerans, Kluyveromyces wickerhamii, Zygosaccharomyces rouxii, Zygosaccharomyces bailii, Zygosaccharomyces cidri, Pichia jadinii or Saccharomyces cerevisiae.

40. The process according to any of claims 28 to 36, wherein the second microorganism is Kluyveromyces marxianus IFO1090, Kluyveromyces thermotolerans ATCC24177, Kluyveromyces wickerhamii ATCC24178, Zygosaccharomyces rouxii NFR2007, Zygosaccharomyces bailii ATCC8766, Zygosaccharomyces cidri ATCC46819, Pichia jadinii IFO0987 or Saccharomyces cerevisiae Kyokai No. 701.

41. The process according to any of claims 28 to 40, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is linoleic acid and the  $\delta$ -lactone is  $\delta$ -decalactone.

42. The process according to any of claims 28 to 40, wherein the straight-chain fatty acid having n carbon atoms (n is an even number of 10 or more) wherein at least the [n-6]-position is a double bond is  $\alpha$ -linolenic acid and the  $\delta$ -lactone is jasmine lactone.

43. The process according to any of claims 28 to 42, wherein the composition is a food.

44. The process for producing a food containing a  $\delta$ -lactone, which comprises adding to a food the  $\delta$ -lactone produced by the process according to any of claims 12 to 27
- 5 or the composition containing the  $\delta$ -lactone produced by the process according to any of claims 28 to 42.

## DECLARATION AND POWER OF ATTORNEY FILED WITH U.S. DESIGNATED OFFICE UNDER 35 U.S.C. 371(c)(4)

\*As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**"PROCESS FOR PRODUCING HYDROXYLATED FATTY ACIDS  
AND  $\delta$ -LACTONES"**

the specification of which was filed as PCT International Application No. PCT/JP00/04535

filed July 7, 2000 and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

Priority Claimed

192684/99	Japan	07 July 1999	<input checked="" type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
			<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
			<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
			<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
			<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)

11  
I hereby appoint as principal attorneys; Donald R. Antonelli, Reg. No. 20,296; David T. Terry, Reg. No. 20,178; Melvin Kraus, Reg. No. 22,466; Stanley A. Wal, Reg. No. 26,432; William I. Solomon, Reg. No. 28,565; Gregory E. Montone, Reg. No. 28,141; Ronald J. Shore, Reg. No. 28577; Donald E. Stout, Reg. No. 26,422; Alan E. Schiavelli, Reg. No. 32,087; James N. Dresser, Reg. No. 22,973 and Carl I. Brundidge, Reg. No. 29,621 to prosecute and transact all business connected with this application and any related United States application and international applications. Please direct all communications to the following address:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Signature

Date

January 7, 2002.